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FINAL REPORT

POSSIBLE ANTAGONISM OF THE BLUESTAIN FUNGUS, CERATOCYSTIS MINOR, BY OTHER MICROORGANISMS ASSOCIATED WITH THE SOUTHERN PINE BEETLE

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POSSIBLE ANTAGONISM OF THE BLUESTAIN FUNGUS, CERATOCYSTIS MINOR, BY OTHER MICROORGANISMS ASSOCIATED WITH THE SOUTHERN PINE BEETLE

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J. Robert Bridges and Thelma J. Perry

INTRODUCTION

The symbiotic association between the southern pine beetle, Dendroctonus frontalis Zimm. (SPB), and the bluestain fungus, Ceratocystis minor, has long been known. The fungus depends on the insect for dissemination and introduction into susceptible trees (Nelson 1934). Whether the beetle benefits from the association has not been unequivocally demonstrated, but bluestain infection causes a reduction in moisture content of the tree and may create conditions that favor brood development (Nelson 1934, Nelson and Beal 1929, Bramble and Holst 1940, Leach et al. 1934). However, direct interaction between bluestain and SPB larvae inhibits beetle development. Where the inner bark has been extensively colonized and stained by C. minor, larvae make atypically long galleries and develop poorly. The life cycle is extended, and complete development may never occur (Barras 1970, Franklin 1970). C. minor does not uniformly colonize the tree. Bluestain is only found in isolated patches. This patchy distribution of C. minor stain is apparently important for SPB development because larval development is inhibited in stained phloem.

The purpose of this study was to investigate the mechanisms that control the patchy distribution of C. minor in bark beetle-infested trees. Specifically, we studied the inhibition of C. minor growth by other microorganisms either by competition for nutrients and space, or by the production of antibiotic compounds.

MATERIALS AND METHODS

Possible antagonists to C. minor were isolated from SPB, its frass, and its galleries. Most of the microorganisms tested were isolated during another study (Bridges et al. 1983).

Agar plate assays--To study interactions on agar plates, C. minor and the candidate microorganism were cultured 20 mm apart on the same plate. Inoculum for fungi was a 3 mm plug of agar from the advancing edge of a 4 to 7 day-old culture on 2.5% malt extract (ME) agar. Bacteria and yeasts were inoculated with a 3 mm transfer loop in a streak 10 mm long. Antagonism studies were done primarily using 2.5% ME agar. Sabouraud's dextrose agar (Difco), potato dextrose agar (Difco), yeast morphology agar (Difco), and wort agar (Difco) were also used.

After 5 to 7 days the distance C. minor grew toward the test organism was measured. C. minor growing on a plate alone was the control. Each test was repeated three times. A positive test the production of antibiotic compounds was the presence of a zone of no growth between the 2 microorganisms.

Bolt assays--Assays were also run to test organisms for antagonism to C. minor in the host tree. Spore suspensions of C. minor and the test organisms were used to inoculate 40 cm loblolly pine bolts in the laboratory. A No. 4 cork borer was used to cut 6 inoculation holes in each bolt.

The inoculation points were equidistant from either end of the bolt and equally spaced around the bolt. Half of the inoculation points received C. minor plus the test organism. The other points received C. minor along as controls. After inoculation the holes were sealed with sterile paraffin. After three weeks the outer bark was removed, and the distance that C. minor grew in the inner bark was measured. Each test was repeated three times. A t-test was used to evaluate differences between the two treatments.

To prepare the spore suspension inoculum, two ml of sterile 0.85% NaCl was pipetted onto the surface of an agar plate of C. minor or test organism and shaken to suspend the spores or cells. The spore concentration was determined with a Newbauer counting chamber. The spore suspension was diluted with saline to produce a suspension with approximately 10^6 spores/ml. Inoculum for C. minor and each test organism contained an equal concentration of spores.

Additional experiments were conducted to investigate the distribution of stain in SPB galleries following inoculation of C. minor into bolts by SPB. The purpose of these experiments was to determine the effects of the SPB microbial complex on C. minor distribution.

SPB preparation--Two treatment groups of SPB were used in the experiment. Both groups were inoculated with C. minor. One group was otherwise fungus-free; the other group was emergent SPB with their normal complement of microorganisms. SPB were obtained from a colony maintained in the laboratory for the purpose of providing a continuous supply of beetles for laboratory experiments.1/ To obtain fungus-free SPB, pupae were collected

1/Bridges, J. R. and J. C. Moser. 1983. A technique for mass rearing the southern pine beetle in the laboratory. Manuscript in preparation.

and surface sterilized in modified White's solution (Barras, 1972). They were then placed in an artificial diet described by Bridges (1979), modified by the addition of antimicrobial agents as described by Marler and Barras (1978). Seven days later fungus-free adults were removed from the diet.

Bolt preparation--A loblolly pine (Pinus taeda L.) 9 inches in diameter breast height was felled and cut into 6 bolts that were 24 inches long. The ends of the bolts were sprayed in the field with modified White's solution (Barras 1972). The entire surface of the bolts were sprayed in the laboratory with a fungicidal solution consisting of a mixture of Benlate and Botran (25 g of each in 750 ml of water). Four sterile nails were driven in one end of each bolt to support the bolt and allow for air circulation when the bolts were stood on end.

Three inches from the top of each bolt a row of corregated joint fasteners (1 x 3 inches) were driven through the bark into the sapwood to completely encircle the bolt. Rows of joint fasteners were placed every 1½ inches along the bolt to form 12 sections bounded on each side by joint fasteners. This method was chosen to alter the normal pattern of beetle gallery construction. When beetles were inoculated into the isolated bolt sections, they were forced to construct galleries around the bolt. This prevented galleries from crossing other galleries. This technique also insured that beetles would construct galleries away from the gallery origin into uninfested phloem. Circling of beetle galleries into phloem already colonized by C. minor was prevented. Figure 1 is a photograph of a bolt before inoculation showing how it was sectioned.

Emergent SPB were from the same colony and were collected on the day of inoculation into bolts.

Fungus preparation--Cultures of C. minor were grown on 2.5% malt extract for 2 weeks. Actively growing colonies of C. minor in which perithecia were exuding ascospores were used. Both groups of beetles (fungus-free and emergent) were inoculated with C. minor by allowing them to walk on a plate of C. minor for approximately 2 hours before inoculation into bolts.

Inoculation of bolts--Using sterile forceps female SPB were inoculated head first into prepared holes. Holes were made in bolts with a No. 4 casing nail. A flamed piece of masking tape was used to cover the hole. Males were introduced one day after females.

Measurement of bluestain--Four weeks after inoculation, the outer bark was removed from bolts to expose SPB galleries. Total length of each gallery and length of gallery with stain were measured. Mean length of gallery, mean length of gallery with stain, and mean proportion of the total gallery with stain were determined. Treatment differences were evaluated with the use of a t-test.

RESULTS AND DISCUSSION

Using the agar plate assay, over 450 microorganisms were tested for antagonism to C. minor. Most of the organisms tested were yeasts, because yeasts are the most frequently isolated microorganisms from SPB (Bridges et al., 1983). None of the yeasts produced antibiotic compounds that inhibited C. minor growth. Several species of hyphal fungi and some bacteria were isolated that were consistently angatonistic to C. minor on agar plate assays. Table 1 is a list of antagonistic organisms isolated in this study.

Table 2 lists of the results of antagonisms in bolts where C. minor and the candidate antagonist were inoculated together at the same inoculation point. These results indicate that some organisms, especially two species of bacteria, significantly slowed the growth of C. minor. However, results of antagonistic interactions of C. minor and other microorgnaism in bolts were inconclusive. Growth of C. minor in phloem tissue was highly variable and measurements were hard to make. Some of the most potent inhibitors from agar plate assays gave variable results in bolt assays. For example, Streptomyces sp gave strongly positive results on agar plate assays, but showed no inhibition of C. minor in bolts (Table 2).

Based on the results of the agar plate assays and bolt assays, the production of antibiotic compounds by symbiotic microorganisms does not appear to be the mechanism controlling the distribution of C. minor. Although antagonists can be isolated from SPB and its galleries, none of the organisms were found frequently enough to explain C. minor distribution based on antimicrobial production by these associated organisms.

Results of experiments of inoculations of bolts with beetles carrying C. minor indicate that the spread of C. minor in galleries is influenced by other microorganisms. These results are presented in Table 3. Of the 36 pairs of beetles in each treatment, 28 from the microbe-free treatment and 30 from the control treatment constructed successful galleries. A successful gallery was one that was greater than 5 cm in length and contained egg niches. Beetles without microorganisms constructed significantly ($P<0.01$) shorter galleries. But, the length of gallery with stain was significantly greater ($P<0.01$) for beetles without the microbial complex. In galleries made by beetles without microorganisms, 91.1% of the gallery had bluestain, whereas, only 27.8% of the gallery was stained in galleries constructed by beetles with symbiotic microorganisms.

In galleries made by microbe-free beetles, stain covered most of the gallery. In galleries made by beetles with symbiotic microorganisms, stain was generally limited to the gallery origin (Figure 2). Nelson (1934) found that 97% of bluestain originated at gallery entrances. Thus, the amount of stain in the galleries was dependent on the presence of the microbial complex. With symbiotic microorganisms present, C. minor did not spread along the gallery indicating that the establishment of C. minor was inhibited by the microbial complex.

The significant decrease in the amount of gallery made by beetles without the microbial complex indicate that the phloem was unsuitable for gallery construction. This unsuitability was probably due to the large amount of bluestained phloem. Beetles have been observed to avoid bluestained areas (Barras, 1970). In the absence of mycangial fungi and C. minor, gallery length was not affected (Barras 1973). In the treatment without microbial complex, SPB females laid eggs. These eggs hatched, but larvae made atypically long larval mines in stained phloem as reported before (Barras 1970; Franklin 1970).

From our data it is is impossible to say which of the microorganisms of then microbial complex is responsible for inhibiting C. minor. Nor is it possible to say how C. minor is inhibited. Although specific antagonism between C. minor and the SPB microbial complex cannot be excluded, the most likely explaination is simply a competition for nutrients and space. We speculate that the mycangial fungi are probably the most important competitors of C. minor. Although yeasts are found in high numbers in SPB galleries (Bridges et al. 1983), the mycangial fungi are known to be obligate symbionts of SPB. Because these fungi are propagated in the mycangium, there is a continual supply of inoculum. These fungi would be inoculated in the gallery as it is being constructed. Once the fungi have become established in the gallery, they probably utilize the available nutrients and space and, thus, limit the growth of C. minor. Whitney and Cobb (1972) suggested that C. minor and the non-staining symbionts of D. brevicomis are mutually exclusive.

C. minor is not carried in the mycangium. It is transported on the exterior of the beetle and by phoretic mites (Bridges and Moser 1983).

The Dendroctonus and Ips species that do not possess a mycangium have a more intimate and compatible relationship with other Ceratocystis species than those that do (Yearian et al. 1972; Whitney 1971). No antagonism between these Ceratocystis species, the associated yeasts, and their beetle host have been recored.

CONCLUSIONS

We conclude our results that the mechanisms that limits the distribution of C. minor in SPB infested trees involves the symbiotic

microorganism complex. We propose another role for symbiotic microorganisms in SPB development. The role would be to control the distribution of C. minor in trees. If there is too little C. minor in the tree, conditions within the tree may not be optimal for brood production (Leach et al 1934; Caird 1934; Nelson 1934). If there is too much C. minor in the tree, brood development will be impaired (Barras 1970). Other roles proposed for the microbial complex include improving the nutritional quality of the phloem (Barras and Hodges 1969; Hodges et al. 1968) and pheromone production (Brand et al. 1976; 1977).

Changes in the SPB microbial complex could affect the amount of C. minor found in phloem. The amount of C. minor present in trees could affect SPB development. Changes in the microbial complex are known to occur. Bridges (1983) found that the mycangial fungi in a population varied from infestation to infestation. Observations of differences in C. minor in trees have also been made between epidemic and endemic populations (Billings 1978; Hetrick 1949).

Table 1. Microorganisms that inhibited the growth of C. minor on agar plate assays by producing antimicrobial compounds.[1]

Bacteria	Hyphal fungi
4A (Unidentified bacterium)	Aspergillus sp.
	Gliocladium sp.
	Penicillium spp.[2]
	Streptomyces spp.[2]
	Trichoderma spp.[2]

1 Assays positive for antimicrobial production were those in which there was a zone around the microorganism with no C. minor growth.

2 Two species.

Table 2. Growth of *C. minor* in a loblolly pine bolt when inoculated alone or together with other microorganisms.[1]

Microbe No.	Microbe type	Growth of *C. minor* with other organism (mm)	Growth of *C. minor* alone (mm)
314381	Yeast	35*[2]	165
324321	Yeast	98*	136
314721	Yeast	61	94
324231	Unknown	100	280
334801	*Streptomyces* sp.	142	153
334691	*Streptomyces* sp.	146	115*
314561	Bacterium	120	152
324311	Yeast	114	122
--	*Penicillin* sp.	107*	132
314412	Bacterium	3*	101
324281	Bacterium	3*	135

[1] Mean of three replications.

[2] * indicates significance at the 0.05 level.

Table 3. Effects of SPB microbial complex on bluestain in SPB galleries.[1]

Treatment	No. successful galleries	Mean gallery length (cm)[2]	Mean length of gallery with stain[2]	Mean proportion of gallery with stain (%)[3]
Without microbial complex	28	19.6	16.96	91.1
With microbial complex	30	38.1**	9.65**	27.8**

1 ** indicates significance at the 0.01 level.

2 Gallery length measurements were transformed using a log transformation before analysis.

3 Percentages were transformed using (X+0.5) before analysis.

LITERATURE CITED

Barras, S. J. 1970. Antagonism between Dendroctonus frontalis and the fungus, Ceratocystis minor. Ann. Entomol. So. Amer. 63:1187-1190.

Barras, S. J. 1972. Improved White's solution for surface sterilization of pupae of Dendroctonus frontalis. J. Econ. Entomol. 65:1504.

Barras, S. J. 1973. Reduction of progeny and development in the southern pine beetle following removal of symbiotic fungi. Can. Ent. 105: 1295-1299.

Barras, S. J. and J. D. Hodges. 1969. Carbohydrates of inner bark of Pinus taeda as affected by Dendroctonus frontalis and associated microorganisms. Can. Ent. 101:489-493.

Bramble, W. C. and E. C. Holst. 1940. Fungi associated with Dendroctonus frontalis in killing shortleaf pines and their effect on conduction. Phytopath. 30:881-899.

Bridges, J. R. 1979. An artificial diet for rearing the southern pine beetle, Dendrocontus frontalis Zimm. (Coleoptera: Scolytidae). J. Georgia Ent. Soc. 14:278-279.

Bridges, J. R. 1983. Mycangial fungi of Dendroctonus frontalis (Coleoptera: Scolytidae) and their relationship to beetle population trends. Environ. Entomol. 12:858-861.

Bridges, J. R., J. E. Marler, and B. H. McSparrin. 1983. A quantitative study of yeasts and bacteria associated with laboratory-reared southern pine beetle larvae. Unpublished manuscript.

Bridges, J. R. and J. C. Moser. 1983. Role of two phoretic mites in transmission of bluestain fungus, Ceratocystis minor. Ecol. Entomol. 8:9-12.

Caird, R. W. 1935. Physiology of pines infested with bark beetles. Bot. Gaz. 96:709-733.

Franklin, R. T. 1970. Observations on the bluestain-southern pine beetle relationship. J. Georgia Entomol. Soc. 5:53-57.

Hetrick, L. A. 1949. Some overlooked relationships of southern pine beetle. J. Econ. Ent. 42: 466-469.

Hodges, J. D., S. J. Barras, and J. M. Mauldin. 1968. Amino acids in the inner bark of loblolly pine, as affected by the southern pine beetle and associated microorganisms. Can. J. Bot. 46:1467-1472.

Leach, J. G., L. W. Orr, and C. Christensen. 1934. The interrelationships of bark beetles and blue-staining fungi in felled Norway pine timber. J. Agric. Res. 49:315-341.

Marler, J. E. and S. J. Barras. 1978. A simple method for the production of microbe-free southern pine beetles. J. Georgia Entomol. Soc. 13:121-124.

Nelson, R. M. 1934. Effect of bluestain on southern pines attacked by bark beetles. Phytopath. Z. 7:327-353.

Nelson, R. M. and J. A. Beal. 1929. Experiments with bluestain fungi in southern pines. Phytopath. 19:1101-1106.

Texas Forest Serv., Texas A&M Univ. 1978. Texas Forest Pest Activity 1976 -1977 and Forest Pest Control Section Biennial Report. Publ. No. 117, 28 pp.

Whitney, H. S. 1971. Association of Dendroctonus ponderosae (Coleoptera: Scolytidae) with blue stain fungi and yeasts during brood development in lodgepole pine. Can. Entomol. 103:1495-1503.

Whitney, H. S. and F. W. Cobb, Jr. 1972. Non-staining fungi associated with Dendroctonus brevicomis (Coleoptera: Scolytidae) on Pinus ponderosa. Can. J. Bot. 50:1943-1945.

Yearian, W. C., R. J. Gouger, and R. C. Wilkinson. 1972. Effects of the bluestain fungus, Ceratocystis ips, on development of Ips bark beetles in pine bolts. Ann. Entomol. Soc. Am. 65:481-487.

Figure 1. Bolt before inoculation showing how it was sectioned using joint fasteners.

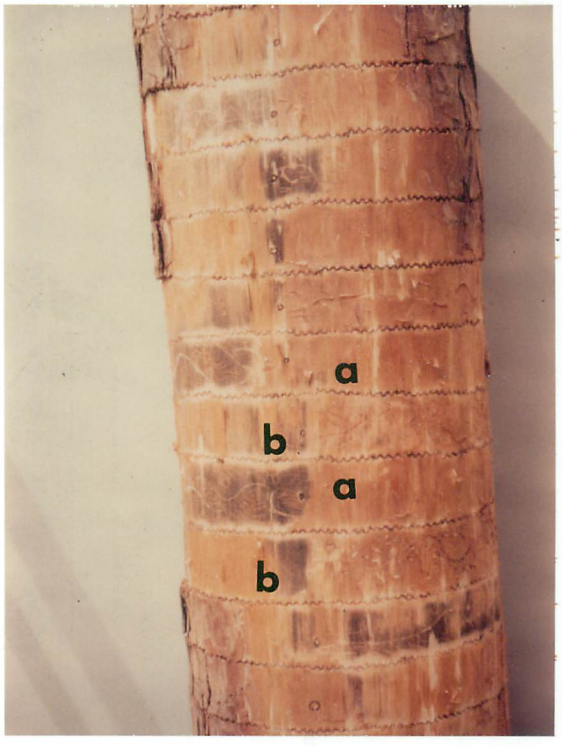

Figure 2. Bolt showing amount of bluestain in galleries made by microbe-free beetles (a) compared to beetles with symbiotic microorganisms (b).